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MOLECULAR STUDY OF SOME VIRULENCE FACTORS AMONG *PSEUDOMONAS AERUGINOSA* RECOVERED FROM BURN INFECTION, IRAQ

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ABSTRACT

During a period of three months (September 2013 – November 2013), a total of 78 Transport swabs with Amies medium were collected from burned victims, who admitted to Burn Unite in **Al**-Hilla General Teaching Hospital. As right practice, all Transport swabs were collected before washing of the burned person and cultured the cetrimide agar plates for selective isolation of *Pseudomonas aeruginosa* isolates. The identification was then confirmed by automated identification VITEK 2 Compact system using VITEK 2GN CARD. Antibiotics susceptibility test was achieved according to CLSI 2012. Genes of some virulence factors like alginate biofilm biosynthesis gene (*algD*) and type III Secretion Toxin genes (*exoS, exoT, exoU, and exoY*) were investigated using specific primers sets by conventional PCR.

The data displayed that (19.2%) of samples gave positive culture for Pseudomonas aeruginosa. All suspected isolates were confirmed by VITEK 2 Compact system. Antibiotics susceptibility test results revealed that low level of resistance were showed for Ticarcillin, Amikacin, Piperacillin, Tobramycin, Norfloxacin and Ceftazidime in which the resistance percent were 6.6%, 6.6%, 6.6%,13.3%, 13.3% and 13.3% respectively. The resistances to other antibiotics were 26.6% for Netilimicin, 66.6% for Nalidixic acid, 80% for Cefuroxime, 86.6% for Cefoperazone. The results of genotypic investigation of some virulence factors gene revealed that, alginate biofilm biosynthesis gene (*algD*) were positive for (53.3%). Type III Secretion Toxin genes result were as follow: *exoS* (80.0%), *exoT* (26.6%), *exoU* (86.6%), *and exoY*(73.3%). As conclusion the results display that, P. aeruginosa isolated from burn infection have the ability to form biofilm which may impair entrance of mechanism of action of many antibiotics or make them need concentration more than the usual to do their work. The aminoglycoside still the best choice for treatment. Many isolates have more than one of type III Secretion Toxin which intensifies their pathogenicity.

KEYWORDS: Pseudomonas, Biofilm, Type III Secretion System, Toxin, Virulence

INTRODUCTION

Burns are one of the most common and devastating forms of trauma. In order to minimize morbidity and mortality resulted from serious thermal injury, a specialized care is required. Burn wounds infection is a great problem because it may lead to death in 75% of patients with injuries [1]. The undamaged human skin surface is vital to the safeguarding of body fluid homeostasis, thermoregulation, and the host's protection against infection. As the first line of defense, the skin is equipped with arange of immune mediators capable of engaging inflammatory cells to support neutralization and clearance of microbes [2].

Following burn injury, bacteria rapidly colonize open skin wounds. Hence, immune failure in a burn patient who has lost the skin barrier is vulnerable to infection[3, 4]. The wound surface is a high protein medium involving of a vascular necrotic tissue that offers a suitableplace for microbial colonization and proliferation [5, 6]. A compromised

migration of immune cells and limitation of systemically administered antimicrobial agents to the area resulted from a vascularity may delay the wound healing [7].

In time, wounds are consequently inhabited with Pseudomonas aeruginosa, and yeasts derived from the host's normal gastrointestinal and upper respiratory flora and with those from hospital environment or via hands of health care persons [8].Even though Staphylococcus aureus remains a common causative agent of wound infection, P. aeruginosa from the gastrointestinal flora and environmental source is the most common cause of burn wound infections in many centers [8, 9].

Alginateis a constituent of the surface of P. aeruginosa that facilities adhesion. The genetic cluster responsible for alginate biosynthesis called algACD. The expression of algD, that encodes the enzyme GDP mannose dehydrogenase, is frequently used as an indicator of the expression of the other genes responsible for biosynthesis of alginate, which is a primary compound of the organic polymer matrix that involves biofilm in P. aeruginosa [10]. The biofilms play a vital role as preclude the action of antimicrobial agents and the host immune system, leads toluxuriant microbial inhabitation at the site of biofilm formation [11]. In vivo, many issues may delay biofilm formation such as microbial nutrient replenishment, exposure to killing by the immune system, and immediate wound cleansing [12].

Many virulence factors (such as alginate, flagella and type III secretion system toxins) produced by P. aeruginosa may mediatesadhesion, immune system evasion, leukocyte killing, tissue destruction, and bloodstream invasion [13]. It also carries many intrinsic and acquired antimicrobial resistance traits that make infected burn wounds difficult to treat [14]. The type III secretion system (T3SS), (Also known as exoenzyme S) is a highly specialized protein secretion apparatus that facilitates the translocation of effector proteins from the bacterial cytoplasm directly into host cells. Along with biofilm formation and quorum sensing, the T3SS is one of the major virulence factors of P. aeruginosa [15]. Through the T3SS, this human pathogen secretes and injects four known effectors into host cells: ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT are closely related functional proteins with N-terminal GTPase activating protein (GAP) activity and C-terminal ADP ribosylase (ADPRT) activity [16]. The GAP domains of both ExoS and ExoT are responsible for the disruption of the actin cytoskeleton, the inhibition of bacterial internalization into epithelial cells and macrophages, the induction of host cell rounding, and the prevention of wound healing [17]. Exoenzyme U (ExoU) is a necrotizing toxin with phospholipase activity, and ExoY is an adenylate cyclase [18]. The other two known effectors are ExoS and ExoT, highly homologous to each other, having a carboxy-terminal ADP-ribosyltransferase (ADPRT) domain and an amino-terminal GTPase-activating (GAP) domain [19].

This study focused on investigation of *algD*, the key of alginate biofilm for, and Type III secretion system toxins among local isolates of P. aeruginosa in Hillacity, Iraq

MATERIALS AND METHODS

Sampling and Identification

Seventy eight transport swabs with Amie's medium were collected from burned victims, who admitted to Burn Unite in Al-Hilla General Teaching Hospital during a period from September 2013 to November 2013. As right practice, all Transport swabs were collected before washing of the burned person and cultured the cetrimide agar plates for selective isolation of *Pseudomonas aeruginosa* isolates. The identification was then confirmed by automated identification VITEK 2 Compact system using VITEK 2GN CARD.

Antimicrobial Susceptibility Test (AST)

Muller-Hinton agar plates were seeded (by spreading) with the P. aeruginosa isolates and the antibiotics discs then applied according to CLSI (2012). It was performed using a pure culture of previously identified bacterial isolates .The inoculums to be used in this test was prepared by adding growth from 2-5 isolated colonies grown on cetrimide agar plates(Himedia/India) to 2.5ml of nutrient broth. This culture was then incubated (in shaker incubator) for (3-4 h) to produce a standard bacterial suspension of moderate turbidity equal to McFarland standard tube (0.5). The growth was then approximately standardized to 0.5 McFarland and sterile swab was used to obtain inoculums from the standardized culture. This inoculum was spread on a Muller-Hinton plate. The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps. Incubation was usually overnight at 37ºC. Antibiotics inhibition zones were measured. Zone sizes were compared to standard to determine the susceptibility or resistance of organism to each antibiotic according to (CLSI, (2012)[20] criteria.

DNA Extraction

Bacteria were grown overnight at 37°C in Luria-Bertani medium (Himedia/India) and the DNA extraction was performed according to the protocol of genomic DNA purification provided by the manufacturing company (Solgent / Korea).

Multiplex PCR

The sequence of the primer pairs sets used in this study were mentioned in the table (1). They include primers sets (Bioneer/Korea) used to amplify algD, exoS,exoT, exoU and exoY genes. The primers were dissolved in Nuclease free water to get the stock primer solution at concentration equal to 100 picomole/microliter. Up on work the stock soltion were diluted with nuclease free water to get the working primer solution equal to 10 picomole/microliter. Two mixture of Multiplex PCR were done using 2X Multiples PCR premix (Solgent/Korea). The 1st. mixture contains algD, exoS and exoT primers pair while the 2nd. mixture contains exoU and exoY primers pair. The amplification condition and mixture components were mentioned in table (2) and (3) respectively.

Table 1: Sequence of the Primers Pair and Amplicon Size

Gene	Primer sequence (5'-3')	Amplicon	Reference
algD	VIC1: 5'-TTCCCTCGCAGAGAAAACATC-3'	520 bp	[21]
	VIC2: 5'-CCTGGTTGATCAGGTCGATCT-3'		
exoS	ExoS-MP5: 5'-GCG AGG TCA GCA GAG TAT CG-3'	118 bp	[22]
	ExoS-MP3: 5'-TTC GGC GTC ACT GTG GAT GC-3'		
exoT	ExoT-MP5: 5'-AAT CGC CGT CCA ACT GCA TGC G-3'	152 bp	
	ExoT-MP5: 5'-TGT TCG CCG AGG TAC TGC TC-3'		
exoU	ExoU-MP5 :5'-CCG TTG TGG TGC CGT TGA AG-3'	134 bp	
	ExoU-MP3: 5'-CCA GAT GTT CAC CGA CTC GC-3'		
exoY	ExoY-MP5: 5'-CGG ATT CTA TGG CAG GGA GG-3'	289 bp	
	ExoY-MP3: 5'-GCC CTT GAT GCA CTC GAC CA-3'		

Table 2: Amplification Conditions

Steps		Temperature (˚C)	Time	No. of cycles
Step 1	Initial denaturation	94	2 min.	1
Step 2	Denaturation	94	30 sec.	36
	Annealing	58	30 sec.	
	Extension	68	1 min.	

Step 3	Final extension	68	7 min.	1
	Hold	4	upon	1

Table 3: Multiplex Mixture Components

Mixture No.	Components	Volume (µl)
1st	Premix 2X Multiples master mix	15
	Forward primer 1 (10 pmol/µl)	1.5
	Reverse primer 1 (10 pmol/µl)	1.5
	Forward primer 2 (10 pmol/µl)	1.5
	Reverse primer 2 (10 pmol/µl)	1.5
	Forward primer 3 (10 pmol/µl)	1.5
	Reverse primer 3 (10 pmol/µl)	1.5
	DNA template	4
	Nuclease free water	2
	Total volume	30 µl
2nd	Premix 2X Multiples master mix	15
	Forward primer 1 (10 pmol/µl)	2
	Reverse primer 1 (10 pmol/µl)	2
	Forward primer 2 (10 pmol/µl)	2
	Reverse primer 2 (10 pmol/µl)	2
	DNA template	5
	Nuclease free water	2
	Total volume	30 µl

RESULTS

The results of *P. aeruginosa* isolation on cetrimide agar plates were (19.2%)figure (1). The automated identification VITEK 2 Compact system using VITEK 2GN CARD give the identical results and this confirm the validity of the cetrimide agar medium as selective medium for P. aeruginosa isolation.

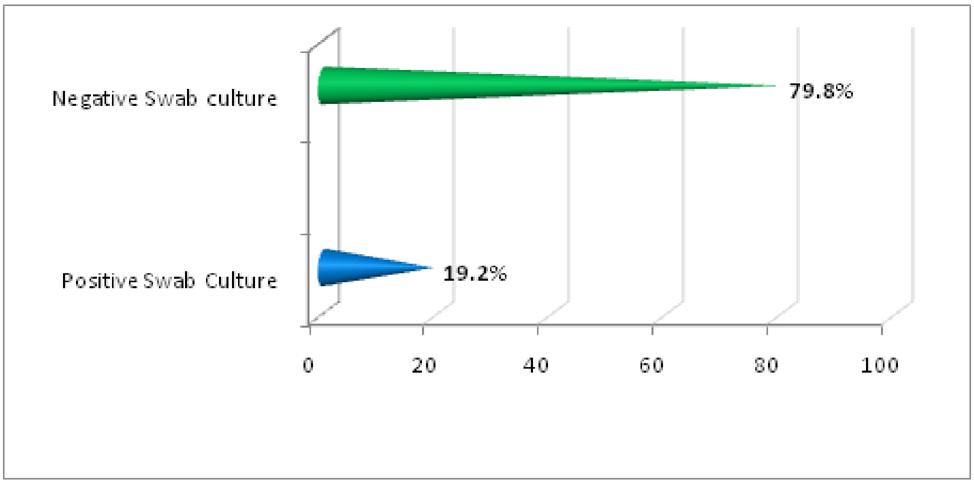

Figure 1: Isolation Percentage of P. Aeruginosa among Burn infection

The results of Antibiotics susceptibility test revealed that all isolates were fully sensitive to polymyxin B and low level of resistance were showed for Ticarcillin, Amikacin, Piperacillin, Tobramycin, Norfloxacin and Ceftazidime in which the resistance percent were 6.6%, 6.6%, 6.6%,13.3%, 13.3% and 13.3% respectively. The resistances to other antibiotics

were 26.6% for Netilimicin, 66.6% for Nalidixic acid, 80% for Cefuroxime, 86.6% for both imipenem and Cefoperazone figure (2).

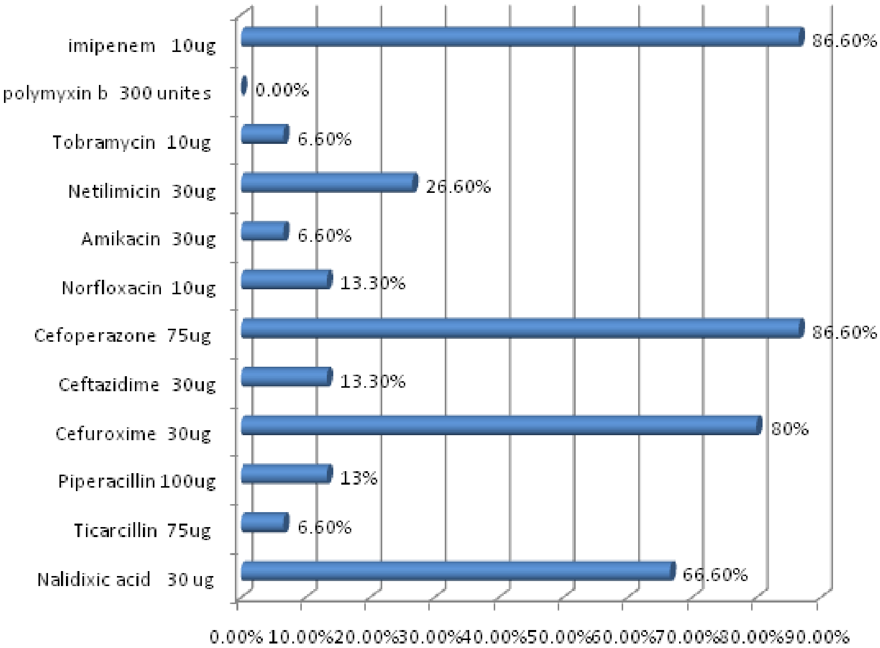

Figure 2: Antibiotics Resistance among P. Aeruginosa Isolates

Concern the alginate biofilm formation the results revealed that 520 bp amplicon of *algD* gene was presents in 7 isolates (53.3%) figure (3). The results of Type III Secretion Toxin genes display that *exoS*was absents in all isolates, *exoT* (80.0%)as mentioned in figure (3). Figure (4) showed that the percentage of the presence of *exoU and exoY* were (86.6%) and *(*73.3%) respectively.

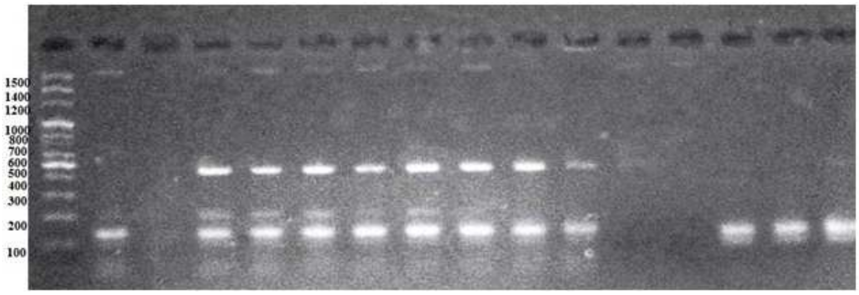

Figure 3: 2% Agarose Gel Electrophoresis of 520 bp Product of algD Gene, 118 bp Product of exoS Gene and 152 bp Product of exoT Gene. Lane 1 Represents the 100 bp Ladder (Solgent/Korea), Lane 2-16 Represent the P. Aeruginosa Isolates

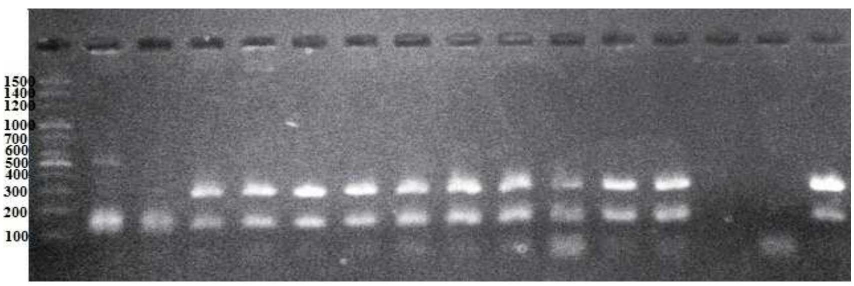

Figure 4: 2% Agarose Gel Electrophoresis of 134 bp Product of exoU Gene, and 289 bp Product of exoY Gene. Lane 1 Represents the 100 Bp Ladder (Solgent/Korea), Lane 2-16 Represent the P. Aeruginosa Isolates

DISCUSSIONS

In this study the results of P. aeruginosa isolation among burn patients was in accordance with those gathered from Bhat and Vinodkumar(2013)[23]Lari and Alaghehbandan(2000)[24] ; Ozumba and Jiburum(2000)[25] and Abbas et. al. (2013) [26] who reported that, P. aeruginosa most common organisms in burn infections and compile 15% - 20% and comes after Staphylococcus aureus. Rezaei et al. (2011) [27] reported that *P. aeruginosa, Acinetobacter, Klebsiella and S. aureus* were the most prevalent organisms isolated from a burn unit in Iran.

The isolation percentage of P. aeruginosa may differ among countries and this might be due to drug abuse, hospital strategy in management and hygiene and geographic climatic [28]. The damaged tissue becomes an ideal culture medium for the growth of microorganisms if not expunged and then grafted. The burn patients are very susceptible to infection because of wide exposed raw areas, the presence of necrotic tissue, protein rich exudates, inability of blood to reach the colonized areas of wounds and other host defense mechanisms [29].

The establishment of infection and later invasion of tissues may be from patient’s normal flora of skin or more usually by cross infection or from gastrointestinal tract. In the face of high mortality because of bacteraemia in burned patients, it is important to select antibiotics or combination of antibiotics with broad coverage for the usual pathogens. In a large number of patients this has to be empirical pending results of cultures [30].

P. aeruginosa displaysinherent resistance to numerous antibiotics, shared with the capability of receiving new resistance information through treatments, through alterations in porins, overexpression of efflux pumps or production of hydrolytic enzymes that damages antimicrobials, such as β-lactamase and metallo-β-lactamases [31]. The carbapenems have been considered the drugs of choice in the treatment of severe nosocomial infections caused by Gram-negative bacteria. However, the isolation of bacteria is already common in Brazilian hospitals, especially P. aeruginosa, resistant to these antibiotics and in these cases polymyxin is the only therapeutic option [32].

The studiedisoaltes showed high sensitivity rates to amikacin, tobramycin, piperacillin and ticarcillin what makes them good choices for treating infections by this pathogen. In agreement to literature data, all strains were susceptible to polymyxin B. Nevertheless, it is indicated for specific situations, due to its toxicity. There are reports about P. aeruginosa strains with reduced sensitivity to this drug [33].

The resistance to imipenem elucidated by several resistance mechanisms, such as loss of proteins of external membrane OprD, that causes resistance to imipenem, superexpression of efflux systems; and carbapenemase production [34].The high antimicrobial resistance may be credited to the unnecessary use of broad spectrum antibiotics and the lack of antibiotic dispensing policy [26].

The alginate play a pivotal role tofunction as an adhesin and in protection of bacteria from mucociliary activity, phagocytosis, complement system activity, decreases the antimicrobial action, making its penetration in the bacterium difficult, and it is also associated to biofilm production [35]. The protection of the bacteria from the host’s immune defense and antibiotics activity may accredited to biofilm formation and the they being more resistant to drugs because the association of cells in the biofilm luxuries the transfer of resistance genes from one bacterium to another through

plasmids.The expression of algD, that encodes the enzyme GDP mannose dehydrogenase, is regularly used as an indicator of the expression of the other genes responsible for biosynthesis of alginate, which is a primary compound of the organic polymer matrix, that involves biofilm in P. aeruginosa and is considered by some authors as responsible for the antimicrobial resistance, observed in biofilm [36, 37].The relative virulence of these effector proteins is important since clinical isolates of P. aeruginosa commonly fall into one of the following three phenotypic categories: (i) those that secrete ExoU and ExoT, (ii) those that secrete ExoS and ExoT, and (iii) those that do not secrete type III proteins [38].In general those strains that secrete ExoU and ExoT may be the most virulent strains. Strains that secrete ExoS and ExoT may be intermediate in virulence. Those strains that are incapable of type III secretion and therefore do not secrete ExoU, ExoS, and ExoT may be the least virulent [39].Many studies stated that, ExoT inhibits cell division and can induce apoptosis in epithelial cells. ExoU has phospholipase A2 activity and causes rapid cell death. ExoY is an adenylate cyclase and may disrupt the actin cytoskeleton. By secreting these effector proteins via the T3SS, P. aeruginosa efficiently inhibits wound repair and the host innate immune response to facilitate its colonization and its ability to cause injury [40].

CONCLUSIONS

Our study conclude that, P. aeruginosa isolated from burn infection have the ability to form biofilm which may impair entrance of mechanism of action of many antibiotics or make them need concentration more than the usual to do their work. The aminoglycoside still the best choice for treatment. Many isolates have more than one of type III Secretion Toxin which intensifies their pathogenicity.

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